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Unusual Expression of mRNA Typical of Philadelphia Positive Acute Lymphoblastic Leukemia Detected in Chronic Myeloid Leukemia

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The Philadelphia chromosome (Ph) is found in both chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). The Ph translocation, t(9;22)(q34;q11), can disrupt the *BCR* gene on chromosome 22 in one to two areas called the major (Mbcr1) and minor (mbcr1) breakpoint cluster regions. In CML the breakpoint has been mapped almost exclusively to Mbcr1, whereas in Ph positive ALL both Mbcr1 and the upstream mbcr1 breakpoints have been described. In this communication we describe an unusual patient with typical chronic phase Ph positive CML and evidence of the uncharacteristic mbcr1 breakpoint, predicting expression of the ALL-type p190 fusion protein. Fluorescence in situ hybridization demonstrated *BCR* gene rearrangement, the reverse transcription polymerase chain reaction detected the *BCR-ABL* fusion mRNA characteristic of the mbcr1 breakpoint, and failed to detect *BCR-ABL* mRNA characteristic of the Mbcr1 breakpoint. Southern blot analysis revealed no rearrangement in Mbcr1, and direct sequencing of the PCR product confirmed it to be the ALL-type mbcr1 fusion mRNA with the first exon of the *BCR* gene fused to *ABL* exon a2. This case differs from the previously reported cases of "p190" CML in that the patient presented without abnormal hematopoietic features other than those found in typical CML and provides further evidence that the p190 mRNA is not sufficient to cause an acute rather than chronic leukemia. © 1996 Wiley-Liss, Inc.

Key words: leukemia, -myeloid, -chronic; bcr-abl; Philadelphia chromosome

INTRODUCTION

Chronic myeloid leukemia (CML) is characterized cytogenetically by the presence of the Philadelphia chromosome (Ph) [1], a derivative chromosome 22 occurring as a result of a reciprocal translocation between chromosomes 9 and 22 [2]. In Ph positive CML the t(9;22)(q34;q11) results in disruption of the c-*ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22. In almost all cases of CML, the breakpoint on chromosome 22 is located within a small 5.8 kb region (major breakpoint cluster region 1 or Mbcr1) between *BCR* exons 2 and 3 or 3 and 4 [3]. As a result of fusion with exon 2 of *ABL*, two different RNA message forms, b3a2 and b2a2, may be detected by reverse transcription polymerase chain reaction (RT-PCR). The hybrid *BCR-ABL* gene in CML encodes a 210 kd fusion protein (p210) with increased tyrosine kinase activity, which has been directly implicated as the molecular cause of the disease [4].

Patients with acute lymphoblastic leukemia (ALL) may also carry the Ph but molecular analyses of these patients has demonstrated that the *c-ABL* gene is relocated to chromosome 22 at Mbcr1 (as in CML) in only about 50% of cases [5]. In the remaining cases the break on 22q11 occurs at a more 5′ location within the first intron of the *BCR* gene (the minor breakpoint cluster region 1 or mbcr1) [6–8]. Rearrangement at mbcr1 results in a 7.5 kb *BCR-ABL* message (e1a2) coding for a 190 kd protein (p190). Although it has been suggested that p190 is specific for an acute rather than chronic leukemic process,

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there are no consistent clinical differences between the p190 and p210 types of Ph positive ALL [9].

In CML, breakpoints outside the Mbcr1 are very unusual (<1%) [10] and a break at mbcr1 has rarely been reported [11–16]. In this communication we describe an unusual patient with classic chronic phase CML who was observed to have the *BCR-ABL* fusion mRNA characteristic of the mbcr1 breakpoint. Individual progenitor cells and individual colonies derived from long-term bone marrow culture (LTMC) of patient bone marrow (BM) repeatedly failed to express either b3a2 or b2a2 message as determined by RT-PCR, but did show *BCR* gene rearrangement by fluorescence in situ hybridization (FISH) using a *BCR*-containing yeast artificial chromosome (YAC) probe [17]. However, individual colonies were found to express the e1a2 message typical of ALL when primers specific for the mbcr1 breakpoint were used for RT-PCR. The demonstration of the "p190" *BCR-ABL* breakpoint in a patient with classic CML provides further evidence that the p190 alone is not sufficient to result in an acute rather than chronic leukemia, and that this rare subtype of CML can occur in patients with classic clinical features of CML.

PATIENT AND METHODS

Patient History

The patient (unique patient number 5371) was a 47-year-old male diagnosed with CML in 1989 after presentation with painful splenomegaly. The peripheral blood showed marked leukocytosis with myeloid elements at all stages of maturation. The total white blood cell count was 225×10^9/L with 50% neutrophils, 25% band forms, 8% metamyelocytes, 5% myelocytes, 2% promyelocytes, 5% eosinophils, 3% lymphocytes, and 2% monocytes. The platelet count was 320×10^9/L. The bone marrow was markedly hypercellular with increased myeloid components at all stages of maturation. The karyotype revealed the Ph in all 20 metaphases examined, with no additional clonal cytogenetic abnormalities. No molecular studies for the *BCR-ABL* rearrangement were performed at diagnosis. The patient was treated initially with alpha interferon, then hydroxyurea and underwent autologous marrow harvest 8 months after diagnosis while awaiting the results of an unrelated donor marrow search. The harvested autologous marrow was morphologically completely consistent with CML in chronic phase, and karyotypic analysis of the autologous marrow again revealed the Ph to be the only clonal abnormality in all 20 metaphases examined. The patient eventually underwent an unrelated donor marrow transplant but died of regiment-related toxicity and infection. In a subsequent study of stem cell biology in CML, the autologous marrow was thawed and individual hematopoietic progenitors (as well as myeloid and erythroid colonies derived from hematopoietic progenitors) were analyzed for evidence of *BCR* gene rearrangement by both FISH and RT-PCR. In addition, a sample of stored peripheral blood (PB) from the patient was used for Western blot and to establish an Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell line for further investigation by cytogenetics, FISH, RT-PCR, and Southern blot.

Selection of Primitive Hematopoietic BM Progenitors

Mononuclear cells were obtained after density centrifugation of thawed marrow and enriched for $CD34^+$ cells using magnetic microbeads (Miltenyi Biotec) after staining with HPCA-2 (Becton Dickinson, San Jose, CA, anti-CD34 fluorescein isothiocyanate direct conjugate). The $CD34^+$ fraction was stained with anti-CD38 conjugated to phycoerythrin (Becton Dickinson) and $CD34^+/38^{lo}$ cells were collected using a FACS-II laser flow cytometry system (Becton Dickinson). $CD34^+/38^{lo}$ cells (10^3/ml) were plated onto irradiated allogeneic stroma in LTMC media as previously described [18]. Nonadherent cells were collected weekly from the LTMCs and replated in clonogenic methylcellulose assays in the presence of recombinant growth factors IL-1, IL-3, IL-6, G-CSF, GM-CSF, and Kit Ligand (gifts from Genetics Institute, Cambridge, MA) at 10 ng/ml and erythropoietin (Amgen, Thousand Oaks, CA) (3 U/ml) in Iscove's media. Individual myeloid and erythroid colonies were harvested and tested for *BCR* gene rearrangement by FISH and RT-PCR.

FISH Detection of *BCR* Gene Disruption

Sorted progenitors ($CD34^+/38^{lo}$) and individual colonies generated from these cells were fixed with methanol/acetic acid after cytocentrifugation onto clean glass slides. The EBV transformed cell line was prepared for FISH by standard cytogenetic techniques. The 215 kb YAC, yWPR415 (alias D107F9) [19] containing both the Mbcr and mbcr1 *BCR* breakpoints was digoxygenin-labeled and hybridized as described previously [17]. In normal cells hybridized with this probe, two fluorescent signals are seen in both metaphase and interphase as a result of probe annealing to the intact *BCR* regions on each chromosome 22. In Ph positive CML cells, one of the two signals is split as a result of the t(9;22), and thus three signals are seen in both metaphase and interphase (Fig. 1). Following hybridization and detection at least 100 cells from each sample were evaluated for the presence of *BCR* rearrangement (demonstrated by three fluorescent signals).

PCR Amplification of *BCR-ABL* mRNA

RT-PCR using nested primers [20] was performed on sorted $CD34^+/38^{lo}$ cells immediately following their isolation, and on individual colonies derived from LTMC. Amplification of β2 microglobulin was used to determine RNA integrity [21]. The PCR primers used to detect

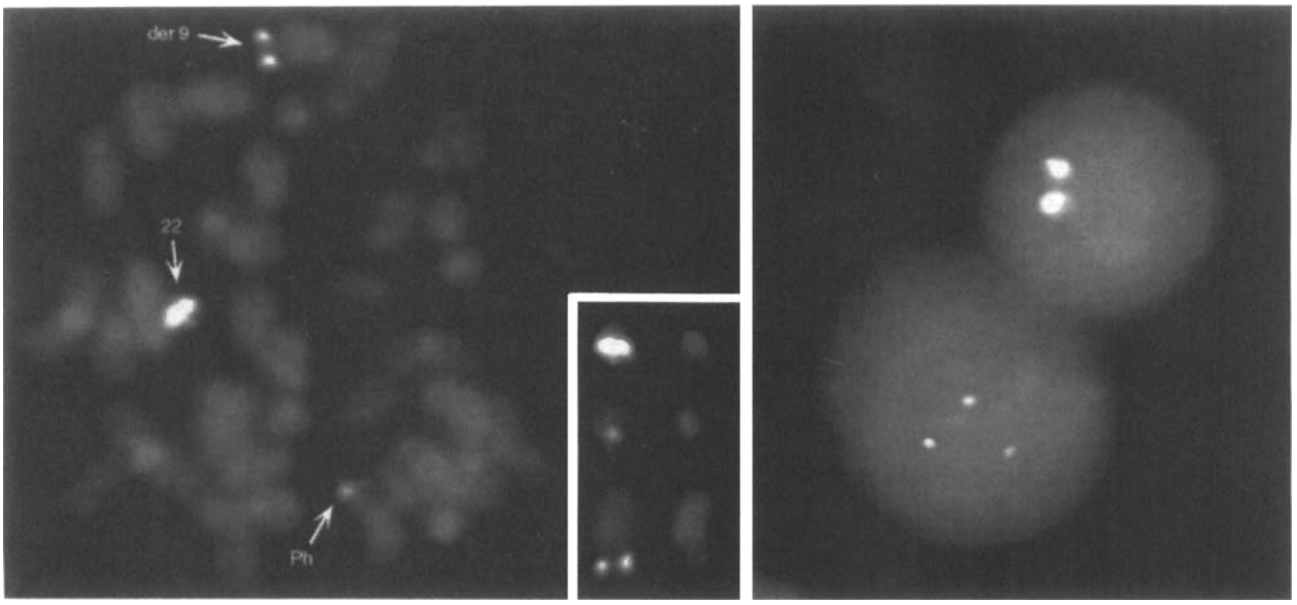

Fig. 1. *BCR* gene rearrangement detected by FISH. Left: A Ph positive metaphase from the EBV transformed patient cell line with FITC (white) signals localized to the BCR gene regions of each chromosome 22 and to the derivative chromosome 9, demonstrated by the arrows. Inset: The individual chromosomes with FITC signal. Right: Cell line interphase nuclei with two signals-normal (right) and three signals-*BCR* rearranged (left).

the Mbcr1 and mbcr1 fusion mRNAs are listed below. Dilutions of 1 pg of total K562 RNA or RNA from a patient with mbcr1 Ph+ ALL into 1 μg HL60 RNA served as positive controls for CML or ALL RT-PCR, respectively. A reaction with all of the PCR components except RNA was used as a negative control. A PCR reaction with methylcellulose media taken from colony assay dishes was used as an additional negative control for experiments on individual colonies to rule out contamination from one colony to another in the process of colony collection.

Primers

1. For Mbcr1:

BCR internal	5′-TGG AGC TGC AGA TGC TGA CCA ACR CG-3′
ABL internal	5′-ARC TCC ACT GGC CAC AAA ATC ATA CA-3′
BCR external	5′-GAA GTG TTT CAG AAG CTT CTC C-3′
ABL external	5′-TGA TTA TAG CCT AAG ACC CGG A-3′

2. For mbcr1:

BCR internal	5′-AGA TCT GGC CCA ACG ATG GCG AGG GC-3′
ABL internal	5′-ATC TCC ACT GGC CAC AAA ATC ATA CA-3′
BCR external	5′-ACC ATA GTG GGC GTC CGC AAG A-3′
ABL external	5′-TGA TTA TAG CCT AAG ACC CGG A-3′

3. For β-2 microglobulin, as published [20].

Southern and Western Blots

Southern analysis of genomic DNA from the EBV-transformed cell line for Mbcr1 rearrangement was performed using the TRANS probe (Oncogene Science, Manhasset, NY) after digestion with the restriction enzymes *Bgl*II and *Xba*1 [10]. Western blot for the detection of p190 and p210 fusion proteins was performed according to published methods [22].

RESULTS

Detection of *BCR* Rearrangement and *BCR-ABL* Transcript in CML Progenitors

In FISH studies of the sorted BM $CD34^{+}/38^{lo}$ population, 60% of interphase nuclei had three fluorescent signals indicating disruption of the *BCR* gene within the 215 kb region spanned by the *BCR*-containing YAC probe. This result was consistent with experiments on 3 other CML Mbcr1 positive BM samples where 47–60%

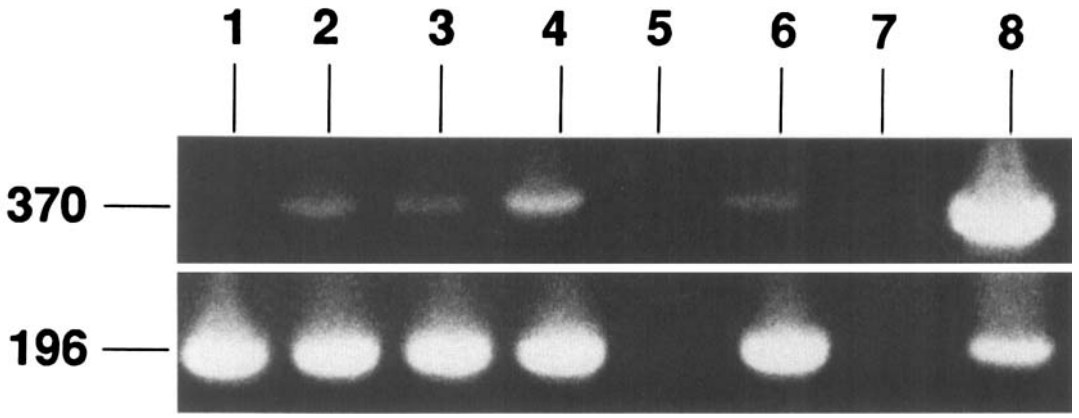

Fig. 2. RT-PCR detection of ALL type *BCR-ABL* mRNA in single myeloid and erythroid colonies. Ethidium bromide stained gel (top) contains bands corresponding to the 370 bp β-2 microglobulin specific product. Bottom: 196 bp bands resulting from PCR amplification of ALL-type *BCR-ABL* mRNA. Lanes contain RT-PCR products as follows: Lanes 1–3: single CFU-GM colonies; lane 4: single BFU-E colony; lane 5: negative control (media only); lane 6: single CFU-GM, lane 7: negative control (H_2O only); lane 8: positive control (Ph^+ $mbcr1^+$ ALL patient).

(mean = 56%) of early CML progenitor nuclei had evidence of *BCR* gene rearrangement by FISH. In normal control samples hybridized with the *BCR* YAC probe, 4.5 ± 1.2% (mean ± SE) of PB (n = 7) and 3.5 ± 0.5% of BM (m = 5) interphase cells had three signals [17]. Despite the observation that *BCR* rearrangement was present in the $CD34^+/38^{lo}$ population as determined by FISH, RT-PCR for the expected CML breakpoints in Mbcr1 was negative. In addition, RT-PCR failed to detect CML type mRNA (b3a2 or b2a2) from unsorted mononuclear cells or from $CD34^+$ cells expressing high or intermediate levels of CD38 antigen from the same patient. Insufficient material was available to also test sorted cells for the presence of the ALL-type mRNA (e1a2) by RT-PCR with alternate primers.

Detection of *BCR* Rearrangement and *BCR-ABL* Transcript in Clonogenic Cells

Following LTMC of $CD34^+/38^{lo}$ cells, individual myeloid and erythroid colonies from secondary clonogenic assays were harvested and tested for *BCR-ABL* rearrangement using RT-PCR with Mbcr1-specific primers. All colonies tested (n = 12) were negative for Mbcr1 *BCR-ABL* message (data not shown), although FISH with the *BCR*-containing YAC on a single large myeloid colony showed that 82% of nuclei from the colony had *BCR* gene rearrangement (three fluorescent signals). Seven additional individual colonies (six myeloid and one erythroid) were subsequently tested for the mbcr1 specific *BCR-ABL* message by RT-PCR (Fig. 2). All colonies demonstrated expression of a message resulting from a fusion of *BCR* exon 1 sequences to *ABL* exon 2, indicating a rare localization of the *BCR* gene breakpoint in the mbcr1 region of this CML patient. The *BCR-ABL* fusion PCR product from one colony was then cloned into a plasmid vector (pT7BlueT-Vector Kit, Novagen, Madison, WI), and fluorescent direct sequencing (Taq Dye-Deoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) confirmed that the PCR product was e1a2.

Having found the e1a2 *BCR-ABL* mRNA associated with p190 expression, Western blot on cryopreserved PB was performed to confirm the presence of p190, but the proteins were found to be degraded (data not shown). The remainder of the stored blood sample had been used to establish an EBV-transformed cell line. The cell line was Ph positive in 3 of 50 metaphase cells by both conventional metaphase cytogenetics and FISH (Fig. 1). Interphase FISH analysis demonstrated 10% of cells in the transformed B-lymphoblastoid cell line to be Ph positive (Fig. 1). Southern analysis of DNA from the cell line showed no detectable rearrangement in the Mbcr1 region and RT-PCR demonstrated exclusively e1a2 expression (data not shown).

DISCUSSION

The Ph chromosome is found in the leukemic cells of 90–95% of patients with CML [1]. In addition, the majority of patients with a complex Ph translocation or Ph negative CML have a similar disruption to the Mbcr1 region [23]. Over 99% of patients with Ph positive CML have a rearrangement in the Mbcr1 region when probes spanning the entire region are used for Southern blot analysis [10].

Some 15–20% of adult ALL patients have a t(9;22) [8,24] which is cytogenetically indistinguishable from the Ph found in CML. Southern blot analysis of cells from these patients identifies a rearrangement of the Mbcr1 identical to that found in CML in up to 50% of cases,

with expression of the same p210 fusion protein. It has been hypothesized that these patients may be presenting with Ph positive acute leukemia following a short or asymptomatic chronic phase of CML [5,6]. The remainder (50–70%) of Ph positive ALL patients also have disruption of the *BCR* gene, but the breakpoint occurs in the first intron of the gene, upstream of the Mbcr1 [8]. As a result, the first exon of *BCR* is fused with *ABL* exon 2, resulting in the transcription of a different 7.5 kb *BCR-ABL* message encoding for a 190 kd protein. It has been suggested that patients with the "p190" breakpoint have a true de novo acute leukemia and that the p190 may be sufficient to drive an acute rather than chronic leukemic process [7,25–27].

In CML, disruption of the *BCR* gene rarely occurs outside the Mbcr1 [10]. Bartram et al. reported the first case of Ph+ CML with a breakpoint 5′ of the Mbcr1 but within the *BCR* gene in a patient during blast crisis [28]. The rearrangement was found to produce the 8.0 kb RNA transcript expected in p210 CML. Several other patients have been described with a breakpoint immediately up or downstream of Mbcr1 [29–32]. When a DNA probe spanning the entire Mbcr1 was used by Blennerhassett et al. for Southern blot analysis of clinical specimens from 191 patients with CML, 190 patients had a rearrangement detected within that region [10].

Selleri et al. described three of 20 patients with chronic phase CML without a breakpoint at Mbcr1 [33]. Subsequently, detailed molecular analysis in two of these cases showed that with the use of more 5′ *BCR* probes, one had deletion of some central *BCR* sequences and the breakpoint was confirmed to be within the Mbcr1. The other patient, however, was found to have deletion of all central *BCR* sequences, and the breakpoint was localized by field inversion gel electrophoresis to mbcr1 with expression of p190 mRNA [11]. Melo et al. recently reported another case of chronic phase Ph positive CML with Mbcr1 germline and expression of ALL type mRNA [16]. In a review of the three other cases reported with clinical details [11,12,15] the authors noted atypical features in all of those cases, with older age, moderate leukocytosis, absence of splenomegaly and monocytosis, suggesting that "p190 CML" may be associated with a phenotype intermediate between CML and chronic myelomonocytic leukemia [16]. The patient reported in our study had none of these clinical features and was also found to express mRNA of the p190 type, with *BCR* exon 1 fused to *ABL* exon 2.

The use of the *BCR*-containing YAC probe for FISH in this case was useful for confirming *BCR* gene rearrangement when PCR primers used to amplify the *BCR-ABL* junctions in p210 CML failed to produce any visible band of amplification. Subsequently, p190-specific primers unexpectedly demonstrated the presence of p190 type mRNA. The breakpoint in a similar patient reported by Selleri et al. was localized by Southern analysis of DNA products after restriction digestion and field inversion gel electrophoresis to be at mbcr1 [11]. We were unable to confirm by DNA analysis that mbcr1 was disrupted. However, this was implied by RT-PCR detection of the p190 type mRNA, with *BCR* disruption shown by FISH and Mbcr1 germline by Southern blot. Alternatively, the breakpoint could have occurred within Mbcr1 as usual in CML, with secondary rearrangements within the *BCR* gene or alternative splicing mechanisms resulting in the expression of the p190 form of *BCR-ABL* mRNA [25]. In either case, this suggests that the p190 type *BCR-ABL* mRNA is insufficient in and of itself to cause an acute rather than chronic leukemic process. Indeed, in this patient the presence of the p190 mRNA seemed to confer a phenotype of typical p210 CML.

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